Research Article

Exploring telomere dynamics in ageing male rat tissues – can tissue-specific differences contribute to age-associated pathologies?

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Abstract

Introduction: Due to increasing life-span, global ageing rates are rising rapidly and age-associated diseases are increasing. To ensure that health-span is concomitant with life-span, a greater understanding of cellular mechanisms of ageing is important.

Methods: Telomere-length analysis from a wide range of tissues from weaning, young adult and middle-aged (3, 12 and 52 week) male Wistar rats were conducted using Southern-blotting. Telomere lengths were compared between tissues and ages using regression models based on the ratios of longest-to-shortest telomere fragments.

Results: Robust linear age-dependent telomere attrition was observed in liver; 3wks vs. 12wks, 3wks vs. 52wks ($p<0.01$), 12wks vs. 52wks ($p<0.05$) and heart; 3wks vs. 12wks ($p<0.05$) and 3wks vs. 52wks ($p<0.001$). More subtle shortening was observed in aorta and epididymal fat; 3wks and 12wks vs. 52wks ($p<0.001$) and in skeletal muscle; 3wks vs. 52wks ($p<0.05$), 12wks vs. 52wks ($p<0.01$). Young thymus telomeres increased in length (3wks vs. 12wks) and then shortened between 12wks and 52wks ($p<0.001$). We also reported disparity in telomere shortening within tissues: Telomeres in ageing brain cortex significantly shortened; 3wks vs. 52wks ($p<0.05$), 12wks vs. 52wks ($p<0.01$). This was not seen in the hypothalamic region. Robust step-wise shortening was observed in renal cortex; 3wks vs. 12wks, 12wks vs. 52wks ($p<0.05$), and 3 weeks vs. 52 weeks ($p<0.001$), which was not as apparent in renal medulla; 3wks vs. 12wks ($p<0.01$) and 3wks vs. 52wks ($p<0.01$). Vastus lateralis skeletal muscle demonstrated the shortest telomere length at weaning and underwent robust age-associated attrition; 3wks vs. 52wks ($p<0.05$), 12wks vs. 52wks; ($p<0.01$). We demonstrated that specific tissues exhibit unique telomere attrition profiles which may partially explain why certain diseases are more prevalent in aged individuals.

Discussion/Conclusion: We show wide variation between tissues in vulnerability to the ageing process. In the future this may help target potential interventions to improve health-span.

Introduction

The World Health Organisation (WHO) estimates that the number of people aged 65 years or older in the world will reach 1.5 billion in 2050, compared with 524 million in 2010 [1]. The ageing population structure has contributed to observed increases in age-associated diseases including cardiovascular disease (CVD), type 2 diabetes (T2D), chronic obstructive pulmonary disease (COPD), cancers, dementia, renal dysfunction, non-alcoholic fatty liver disease (NAFLD) and obesity. These non-communicable diseases (NCDs) are now responsible for 60% of all deaths world-wide [2].

The increase in the ageing population places an enormous strain on healthcare and social care systems throughout the world. Therefore much research is focused on understanding the dynamics and underlying molecular mechanisms of the ageing process. Age-associated disease prevalence is highly varied. For example, the so-called 'big four' NCDs, (CVD, T2D, cancers and COPD) make up almost 30% of all NCDs, when expressed in the terms of disability-adjusted life year (DALY) score; (a measure of overall disease burden) [2]. A major caveat in dissecting out why these four NCDs are the most prevalent of all global NCDs, is understanding how both modifiable environmental factors and inherent differences in the rate of cellular ageing affects specific tissues. In order to address this question, studies of cellular ageing mechanisms in a range of tissues are required.

Telomeres are guanine-rich repeat DNA sequences ($TTAGGG^{n}$) which are situated at the ends of chromosomes and are essential to preserve genomic integrity and stability [3]. The extreme terminus of telomeres comprises a guanine-rich 3' overhang that serves as a template for elongation and forms a telomeric `T-loop' which acts to stabilise the telomere. Most healthy somatic cells do not express or have low levels of the telomere elongation enzyme, telomerase. Telomerase forms a protective 'cap' around the telomere, thereby preventing its shortening. It comprises of two main components; Tert, a catalytic component with reverse transcriptase activity [4] and Terc, which serves as a template for telomeric DNA synthesis [5]. Without telomerase, eukaryotic telomeres shorten with each cellular division, due to the 3' end replication problem in which full extension of a linear chromosome cannot occur. When telomeres of somatic cells reach a critically short length, a conformational change that resembles DNA damage occurs and this triggers cell senescence pathways. Senescent cells by nature are non-proliferating and are known to contribute to ageing related diseases and morbidity [6]. Moreover, several studies have shown that telomere shortening is implicitly

associated with cellular ageing [7], disease pathogenesis [8,9] and lifespan [10,11]. Most importantly, it is evident that accumulation of critically short telomeres is essential in modulating cell senescence and eventual disease pathogenesis [12]. Consequently, we developed a telomere length analysis methodology using a telomere restriction fragment (TRF) analysis in which the TRF smear was divided into four discreet regions based upon molecular weight. This included a very short size range (1.3-4.2kb), intermediate short (4.2-8.6kb), intermediate long (8.6-48.5kb) and very long (48.5-145kb) telomere lengths [13].

Previously we have demonstrated telomere shortening in several ageing rat tissues (liver, lung, pancreas and whole kidney) which was undetectable using the traditional mean whole telomere TRF method of telomere length analysis [13]. Whole brain however, demonstrated no change in telomere length with age, using either method [13]. In humans, it is apparent that an altered pattern of telomere length dynamics occurs across the life-course. In the first years of life, significant age-associated telomere shortening in leucocytes has been reported, albeit with high variability reported between individuals [14]. Leucocyte telomere length then becomes relatively stable throughout later childhood, preadolescent and adolescent years and eventually, telomere shortening rate increases in very old age [15]. Moreover, telomere dynamics between tissues may alter between tissues, which could potentially explain why different systems in the body are more vulnerable to age-associated changes than others.

This study therefore aimed to establish if telomere length in a wide range of rat tissues varied from an early time-point (weaning) to 52 weeks of age. Secondly, we aimed to establish if different tissues exhibited altered rates of age-associated telomere attrition. Within the experimental design we performed both TRF length analysis methods and further interrogated the size distribution analysis method to maximise its sensitivity. These data may identify tissues that are likely to be most vulnerable to external factors that influence the ageing process and therefore why some NCDs are more prevalent than others.

Materials and methods

Male and female Wistar rats (*Rattus norvegicus*) of breeding age (5-8 weeks of age) were purchased from Charles River UK, allowed to acclimatise in Specific Pathogen Free (SPF) housing using individually ventilated cages with environmental enrichment and then mated to generate an in-house breeding colony. Virgin female offspring weaned from the breeding colony were maintained at 22°C, on a controlled 12:12-h light-dark cycle and mated at approximately 6 weeks of age (around 240g body weight). Pregnant rats were maintained at 22°C, on a controlled 12:12-h light-dark cycle, in Specific Pathogen Free (SPF) housing using individually ventilated cages with environmental enrichment. Upon detection of a vaginal plug dams were maintained on a 20% protein control diet (consisting of 22g/100g Casein protein (88g protein/100g) 5.05g/100g mineral and vitamin mixture, 0.22g/100g dI-methionine, 8g/100g maize starch, 5g/100g cellulose, 4.3g/100g soyabean oil, 55.15g/100g glucose) as previously described [16]. Access to the diet and water was provided *ad libitum*. The diet was purchased from Arie Blok (Woerden, The Netherlands). The day of birth was recorded as day 1 of postnatal life. Litters were standardized to 8 pups 48 hours after birth, and 4 males in each litter were used in this study. Animals were weaned at 21 days of age onto a standardized pellet diet (RM3) (consisting of 15.43% fibre, 1.43% pectin, 9.2% hemicellulose, 3.93% cellulose, 1.5% lignin, 33.92% starch, 5.75% sugar, 20.77% assorted amino acids, 4.54% assorted fatty acids, 4.65% macro minerals (Special Diet Services, Witham, Essex). One male in each litter was culled at 3 weeks of age; one at 12 weeks of age, and a third male was culled at 52 weeks of age. All animals were killed by CO_2 asphyxiation at approximately 10am after an overnight fast. At post-mortem, heart, aorta, liver, kidney cortex, kidney medulla, vastus lateralis (VL) skeletal muscle, liver, epididymal fat, thymus, spleen and brain were removed, weighed, and snap frozen in liquid nitrogen and then stored at -80ºC until analysis. The brain was dissected into cortex and hypothalamic regions after freezing. In all cases, n refers to the number of litters used with the mother as the statistical unit and with 1 animal used from each litter at each time point.

Telomere length measurement

High molecular weight genomic DNA was extracted using a Phenol/Chloroform DNA methodology. DNA quantity and purity were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) [17]. DNA integrity was confirmed by agarose gel electrophoresis. DNA (1.2 µg) was digested by *Hinf1* and

Rsa1 restriction enzymes at 37°C for 2 hours, separated by Pulsed Field Gel Electrophoresis (PFGE) and transferred to nylon membranes by Southern Blotting [17, 18]. Standard undigested and digested genomic DNA from a 3 week animal was also included on each gel to verify digestion efficiency as described previously [17]. Telomere length was measured using Telo TAGGG telomere length assays (Roche Diagnostics, Welwyn Garden City, Herts, UK). Telomere signals were analyzed using Alpha Ease Software (Alpha Innotech, Exeter, Devon, UK). Telomere length was measured using the mean TRF methodology, in which the whole telomere smear was quantified. This methodology is detailed in the manufacturers' instructions (Roche Diagnostics, Welwyn Garden City, Herts, UK). This was compared with our method of 'discrete fragment TRF' telomere length analysis, in which the percentage intensity (% telomere length) of the telomeric signal was determined according to 4 molecular size regions, defined by molecular weight markers [17, 18].

Telomere length analysis

Telomere length analysis was performed based on telomere restriction fragments (TRF). We performed a head to head analysis of methods based on the mean TRF, in which the whole telomere smear was quantified, and analysis in which the TRF smear was divided into four discreet regions. The four regions were: very short (1.3-4.2kb), intermediate short (4.2-8.6kb), intermediate long (8.6-48.5kb) and very long (48.5-145kb), and were defined by molecular weight markers [13, 17, 18].

In the initial modelling phase, we compared four different ways of expressing telomere length. These were: i) the mean TRF method, (ii) the weighted mean percentage telomere fragment length in each sample (i.e. the index of all molecular weight 'bins' together), (iii) the percentage of telomeres falling within each fragment-length bin separately, and vi) the ratio of the percentage of very long/very short telomeres (% L/S). We used linear regression models including variables for age and tissue-type to compare the model fit for each method. The optimal model was selected using the Akaike Information Criterion (AIC) and lowest residual values. Using the ratio of long-to-short telomeres (% L/S) gave the best fit to the data overall, particularly when compared with the mean TRF method that is more often used [19]. The % L/S method could detect an overall telomere shortening of combined tissues (shown in Fig. 1 a), which was not possible using the mean TRF method (shown in Fig. 1 b).

Gene expression of components modulating telomere length

RNA was extracted using an RNeasy Plus mini kit (Qiagen, Hilden, Germany) following manufacturers' instructions. A DNase digestion step was performed in order to ensure no gDNA contamination. RNA (1 μg) was used to synthesize cDNA using oligo-dT primers and M-MLV reverse transcriptase (Promega, Southampton, Hants, UK). Gene expression was determined using custom designed primers (Sigma, Poole, Dorset, UK) and SYBR Green reagents (Applied Biosystems, Warrington, Cheshire, UK). Primer sequences are presented in Table 1. Quantification of gene expression was performed using a Step One Plus RT-PCR machine (Applied Biosystems, Warrington, Cheshire, UK). Equal efficiency of the reverse transcription of RNA from all groups was confirmed through quantification of expression of the housekeeping gene *Ppia.* Expression of *Ppia* did not differ with respect to age for any tissues studied (S1 Table).

Statistical analysis

Statistical analysis for telomere length and gene expression was conducted using a 1-way ANOVA with age as the independent variable and Duncan's Post-Hoc testing where appropriate, using Statistica, (Statsoft, Palo Alto, CA, USA). Logistic regression and correction for multiple hypothesis testing was performed using R, version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). Where p values are reported, an alpha level <0.05 was considered statistically significant. Data are represented as mean ± S.E.M.

Results

Morphometric analysis of ageing male rat tissues

As expected, body weights and all tissue weights increased significantly with age (Table 2).

Overall effect of ageing upon telomere length in male rat tissues

Telomere length decreased ($p<0.001$) with age when assessed using the ratio of long-to-short telomeres (shown in Fig. 1 a), but this was not apparent using the mean TRF method (shown in Fig. 1 b).

Overall effect of ageing upon telomerase expression

Although overall *Tert1* and *Terc* expression numerically decreased with age, this was statistically non-significant (*Tert1*; $p=0.24$) (*Terc*; $p=0.22$) (shown in Fig. 1 c and Fig. 1 d).

The effect of tissue type upon telomere length in male rats at weaning

At weaning, telomere length varied by tissue type: The ratio of long-to-short telomeres was highest (approximately 1.0 % L/S) in the kidney (1.14 ± 0.2 % L/S), the liver (1.01 ± 0.04 % L/S), and thymus (0.93 ± 0.08 % L/S) (shown in Fig. 2 a). This was followed by telomere lengths in the spleen (0.73 ± 0.13 % L/S), heart (0.61 ± 0.07 % L/S) and aorta (0.68 ± 0.05 % L/S), the epididymal fat pad (0.48 ± 0.03 % L/S), brain cortex (0.44 ± 0.02 % L/S) and brain hypothalamus (0.54 ± 0.03 % L/S) (shown in Fig. 2 a). Telomeres from vastus lateralis (VL) skeletal muscle were shortest (0.36 ± 0.02 % L/S) (shown in Fig. 2 a).

Telomerase components at weaning

Different tissues had a range of telomerase expression levels at weaning. Detectable levels of *Tert1* and *Terc* was observed in the liver of weanling rats, with lower but still detectable expression of telomerase observed in the kidney, which was also the case for *Tert1* expression in brain cortex and hypothalamus (shown in Fig. 2 b and Fig. 2 c). Heart and aorta were on the margins of detection for telomerase expression. *Tert1* and *Terc* were not detectable in epididymal fat (shown in Fig. 2 b and Fig. 2 c). The immunological tissues (thymus and spleen) had extremely high expression of both *Tert1* and *Terc* (shown in Fig. 2 b and Fig. 2 c) at weaning.

Comparison of age-associated telomere length dynamics

Significant age-associated telomere attrition was observed in the classical insulin sensitive tissues (except brain and kidney): Liver (3 vs. 12 weeks; $p<0.01$, 12 vs. 52 weeks; $p<0.05$, 3 vs. 52 weeks; $p<0.01$), VL (3 vs. 52 weeks; $p<0.05$, 12 vs. 52 weeks; $p<0.01$) and the epididymal fat pad (3 and 12 weeks vs. 52 weeks; $p<0.001$) (shown in Fig. 3 a).

Telomeres from both regions of the kidney shortened significantly with age: Kidney cortex (3 weeks vs. 12 weeks; $p<0.05$, 12 vs. 52 weeks; $p<0.05$ and 3 weeks vs. 52 weeks; $p<0.001$) (shown in Fig. 3 b) and kidney medulla (3 vs. 12 weeks and 3 vs. 52 weeks, $p<0.01$) (shown in Fig. 3 b).

In the cardiovascular system, significant age-associated telomere shortening was observed in both cardiac (3 vs. 12 weeks; $p<0.05$ and vs. 52 weeks, $p<0.001$) (shown in Figure 3 c) and aortic tissue (3 and 12 weeks vs. 52 weeks; $p<0.001$) (shown in Fig. 3 c).

Immunological tissues demonstrated a unique pattern of telomere length dynamics. In the thymus, telomere length elongated significantly in the young tissue then between 12 and 52 weeks decreased significantly back to levels observed at 3 weeks (3 vs. 12 weeks and vs. 52 weeks; $p<0.001$) (shown in Fig. 3 d). In the spleen, no significant age-associated telomere shortening was observed (shown in Fig. 3 d).

The brain cortex demonstrated shorter telomeres at 52 weeks compared to 3 weeks ($p<0.05$) and compared to 12 weeks ($p<0.01$) (shown in Fig. 3 e), however no change in telomere length was observed in the hypothalamic region of the brain (shown in Fig. 3 e).

Discussion

Improved understanding of mechanisms that modulate ageing may ultimately lead to extension of health-span. Telomere length is a robust marker of cellular ageing and disease pathogenesis [7, 11], and is often considered a proxy for biological ageing.

We demonstrate significant shortening of telomeres in ageing tissues that was not detected using the mean TRF method. Our mathematical modelling suggests that measurement of discrete areas of the telomere is the optimum method of telomere length measurement. These findings are particularly important, given that the loss of a few hundred base pairs from short telomeres is pivotal to cellular ageing [11,12]. Such losses may go undetected by traditional mean TRF analysis as the TRFs with few telomeric repeats seem to be obscured by the strong signal from other TRFs with long telomeres.

Weaning telomere length & telomerase expression

Telomeres at weaning were longest in thymus, liver and the kidney. In the thymus, preservation of telomere length is essential to preserve immunological function and T-cell lineage [20] and may be related to the high expression of telomerase in young thymic tissue, as evidenced in this study and in previous work [20,21]. In the rat, longer hepatic telomeres at weaning may also relate in part to the marginally telomerase positive nature of this tissue [22]. This is recapitulated by the observed low, but detectable expression of *Tert1* and *Terc* mRNA. The liver is a regenerative organ and telomerase expression has been linked with the tissue's regenerative properties [23,24]. Therefore rat hepatic telomeres may start longer in early postnatal life to prepare for the potential increased cell division later in life when the liver may need to regenerate. Male rat kidneys are known to deteriorate relatively quickly with age and have increased susceptibility to age-associated end stage renal damage, with renal disease the most common cause of mortality in ageing male rats [25]. Therefore long renal telomeres at weaning may be a compensatory attempt to preserve telomere length and cellular integrity for as long as possible. Preservation of renal telomere length at weaning may be related to the detectable levels of *Tert1* and *Terc* expression. In contrast VL telomeres at weaning demonstrated the smallest proportion of long telomeres suggesting that telomeres in this type of skeletal muscle are already much shorter than in other tissues in early life.

Tissue-specific telomere length

The rates and patterns of age-associated changes in telomere length differed between tissues. All classical insulin sensitive tissues, with the exception of the brain underwent significant telomere shortening with age: The liver underwent a significant step-wise shortening (behaving like a classical somatic cell population). Accelerated hepatic telomere shortening is associated with the progression of liver disease including non-alcoholic fatty liver disease, fibrosis, [26] cirrhosis and hepatocellular carcinoma [27]. The epididymal fat maintained longer telomeres at 3 and 12 weeks of age, which then drops off significantly at 52 weeks of age. In VL, the robust age-associated telomere shortening combined with the fact that this tissue started with the shortest telomeres at weaning, may have particular detrimental implications for the aetiology of glucose intolerance, insulin sensitivity and T2D [28-30].

Subtle differences in the dynamics of age-associated telomere shortening were apparent between regions of the kidney. The renal cortex underwent robust step-wise shortening with significant telomere attrition observed throughout the ageing process. However, the renal medulla underwent a more graded telomere attrition which occurred only between young (3 to 12 weeks of age) tissue. Previously, we demonstrated that the cortex region of rat kidney shortens faster than the medulla [31], which is also reflective of that observed in the human [32]. This may potentially be due to differences in mitochondrial density. Mammalian proximal renal tubules contain an abundance of large mitochondria dependent on oxidative rather than anaerobic metabolism [33]. In contrast, the cells within the inner medulla are known to have a far lower mitochondrial content compared with the cortical cells. Mitochondria are known contributors to reactive oxygen species (ROS) generation [34] which preferentially damages telomeric DNA sequences, due to guanine-rich residues within the telomere [35]. The renal cortex may be more liable to generate ROS via oxidative metabolism than the more anaerobic medullary cells and therefore will have telomeres which shorten faster than those found in the medulla.

We also observed an age-associated shortening of cardiac telomeres, suggesting that this tissue is behaving like a classic somatic cell type. Aortic telomeres also shorten with age, however this began after 12 weeks, with no significant shortening observed in young tissue. Cardiac tissue is extremely metabolically active and cardiomyocytes have a high mitochondrial mass by comparison to other less metabolically active tissues [36]. Conversely, myocytes from smooth muscle (aorta) have relatively few mitochondria (22% of the estimated number in cardiac muscle) [37]. Therefore, it is plausible that the increased

mitochondrial mass in cardiac muscle is a key modulator of the accelerated telomere shortening observed in the heart.

Between 3 and 12 weeks of age, the ratio of long-to-short telomeres in the thymus increased. This pattern was unique to this tissue and may be necessary for development of immunological function [20]. We previously reported that telomere length in the brain did not change in the ageing male rat [13], which has also been reported in human brain tissue [38]. We also observed that age-associated telomere length attrition was much less pronounced in the brain tissue overall, compared to other tissues. Interestingly, when the cortex and hypothalamic regions were analysed separately, telomere shortening was detected in the cortex which was not observed in the hypothalamus. It has been demonstrated that cortical brain regions are more sensitive to development of Alzheimer's disease [39]. Therefore, the observed accelerated telomere shortening in the brain cortex may be a driving factor in its susceptibility to age-associated neurodegeneration which specifically affect cortical regions of the brain.

In conclusion, our findings are consistent with factors relating to telomere length dynamics being pivotal in cell senescence and that the observed patterns vary considerably by tissue type, [40]. Telomere length in early postnatal life (weaning) could be regarded as an important set-point in determining the fate of the tissue in later life, particularly if this is combined with a high rate of telomere length attrition, as demonstrated in skeletal muscle. This is particularly important given recent evidence suggesting that the rate of telomere attrition is a powerful predictor of lifespan [11]. However rapid telomere attrition may occur irrespective of the initial 'set-point' of telomere length, as demonstrated in cardiac tissue and in the kidney in which telomeres were not short at weaning, however they underwent robust rates of age-associated telomere attrition. High mitochondrial density and therefore oxidative stress are perhaps pivotal mechanisms for these findings. Our findings may partially explain why CVD and T2D are common NCDs, however we acknowledge that we cannot claim causality based upon these findings alone. We also demonstrated that the function of the tissue may also be a factor in determining telomere dynamics: The urinary tract, CNS, and immunological tissues demonstrate markedly different age-associated telomere dynamics, potentially driven by the differences in cell proliferation and/or different mitochondrial densities, yet this association needs to be fully elucidated. A greater understanding of telomere dynamics could potentially help determine which tissues are more vulnerable to the

ageing process and help to target any potential interventions. We also demonstrated that our discrete TRF telomere fragment analysis, was the more sensitive and reliable approach for analysing telomere length in a wide range of ageing rat tissues, compared with the mean TRF method in a comprehensive range of rat tissues.

Statement of Ethics

All protocols were approved by the Animal Welfare and Ethical Review Board (AWERB) of the University of Cambridge and carried out in accordance with the U.K Animals (Scientific Procedures) Act 1986 and followed the ARRIVE guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author contributions

JLA; conceptualisation, data curation, formal analysis, investigation, methodology, writing (original draft & review and editing). CEA; investigation, methodology, validation, visualisation, writing (original draft & review and editing). LD; resources, writing (original draft & review and editing). DSFT; resources; writing (original draft & review and editing). SEO; conceptualisation, funding acquisition, project administration, supervision, writing (original draft & review and editing).

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Figure Legends

Fig. 1: The effect of ageing upon overall telomere length measured by Southern Blotting and telomerase expression by RT-PCR in rats at 3, 12 and 52 weeks of age using a) Discrete fragment TRF method or b) Mean TRF method. c) *Tert1* expression and d) *Terc* expression. N=8 per group; ± SEM.

Fig 2: a) Telomere length measured by Southern Blotting, b) *Tert1* expression and c) *Terc* expression measured by RT-PCR in weanling male rats. N=8 per group; ± SEM.

Fig. 3: The effect of individual tissue types upon telomere length measured by Southern Blotting in: a) Insulin sensitive, b) Urinary system, c) Cardiovascular system, d) Immunological system, e) Brain cortex & hypothalamus. N=8 per group; ± SEM.

Fig. 1

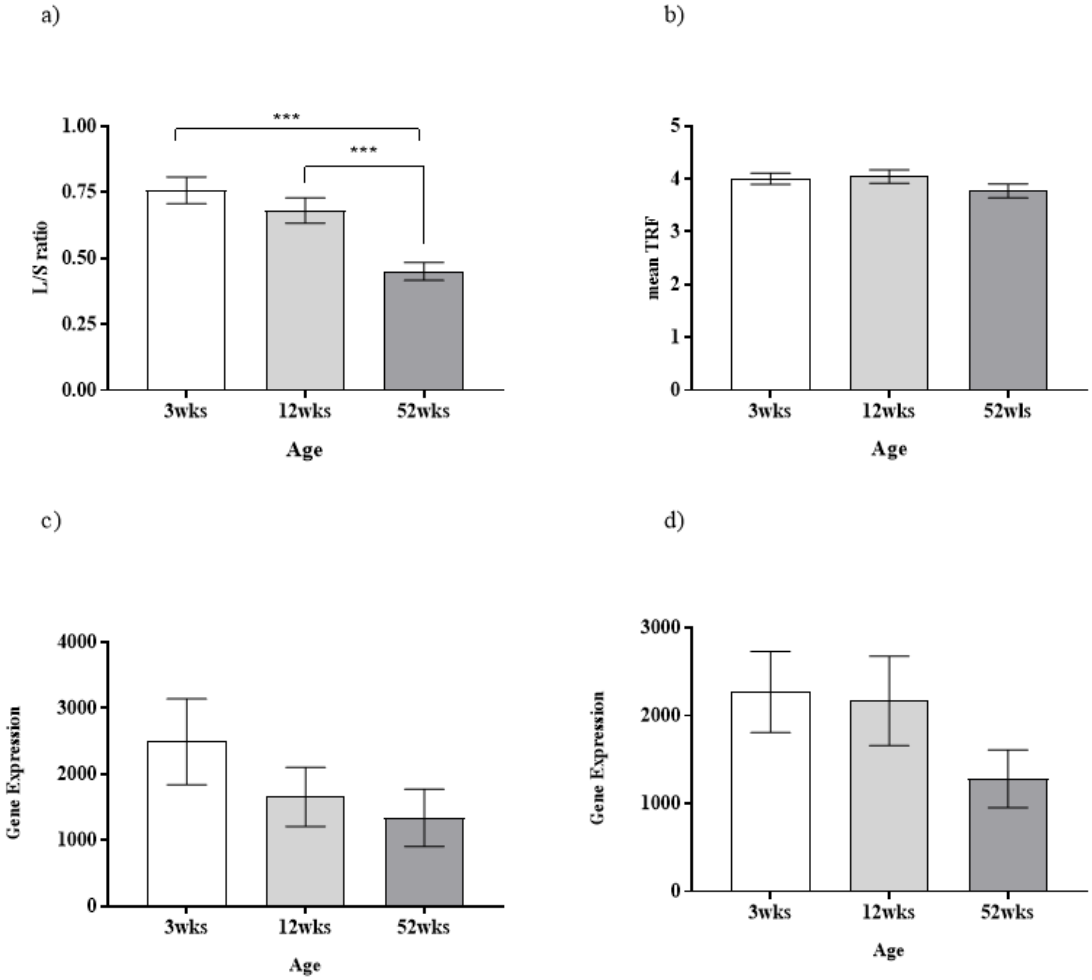
a)
b)
c)
d)
L/S ratio
mean TRF
Gene Expression
Gene Expression
***
***
1.00
0.75
0.50
0.25
0.00
5
4
3
2
1
0
4000
3000
2000
1000
0
3000
2000
1000
0
3wks
12wks
52wks
52wls
Age

Fig. 2

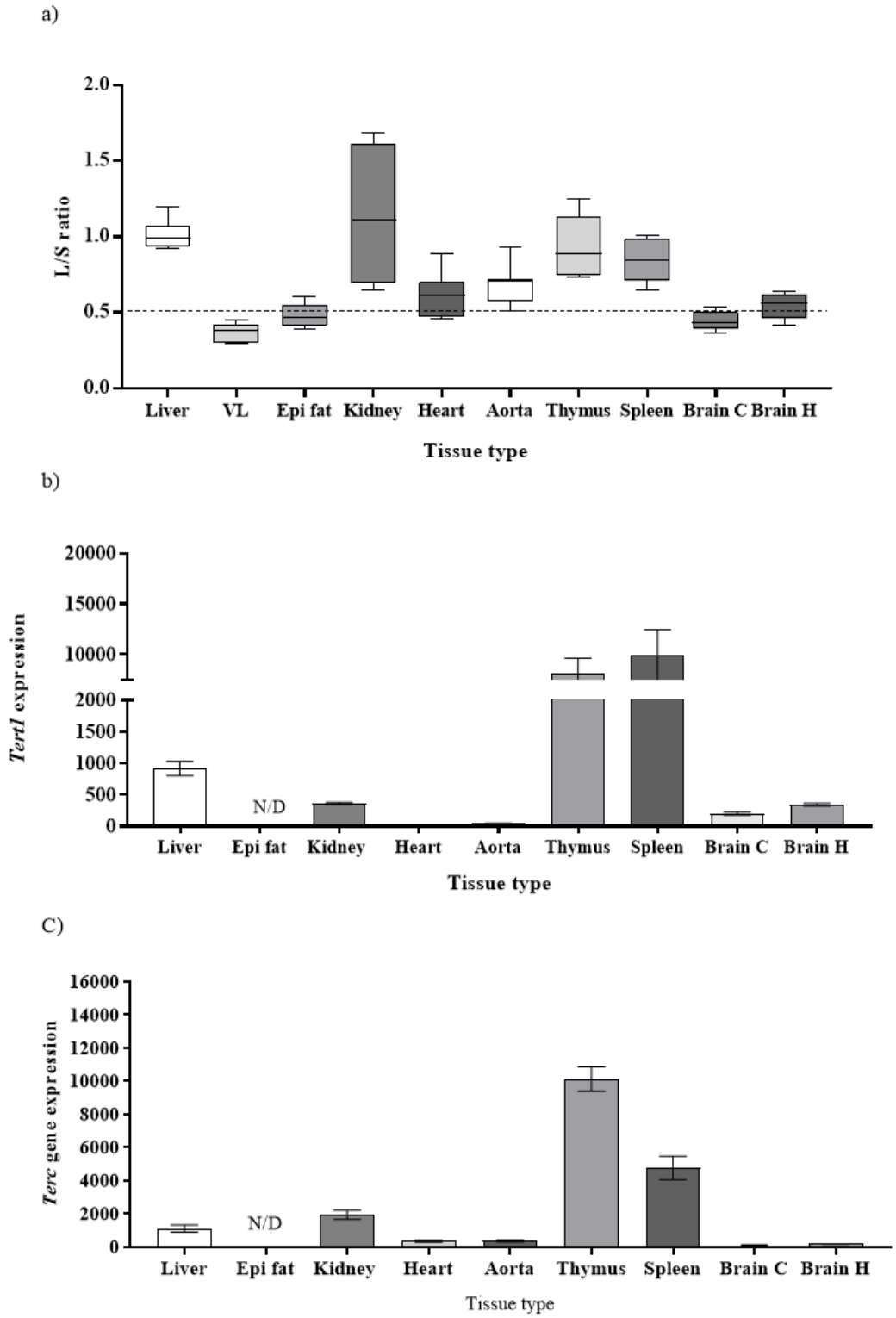
a)
2.0
1.5
1.0
0.5
0.0
L/S ratio
Liver
VL
Epi fat
Kidney
Heart
Aorta
Thymus
Spleen
Brain C
Brain H
Tissue type
b)
20000
15000
10000
2000
1500
1000
500
0
Tert1 expression
N/D
Liver
Epi fat
Kidney
Heart
Aorta
Thymus
Spleen
Brain C
Brain H
Tissue type
C)
16000
14000
12000
10000
8000
6000
4000
2000
0
Terc gene expression
N/D
Liver
Epi fat
Kidney
Heart
Aorta
Thymus
Spleen
Brain C
Brain H
Tissue type

Fig. 3

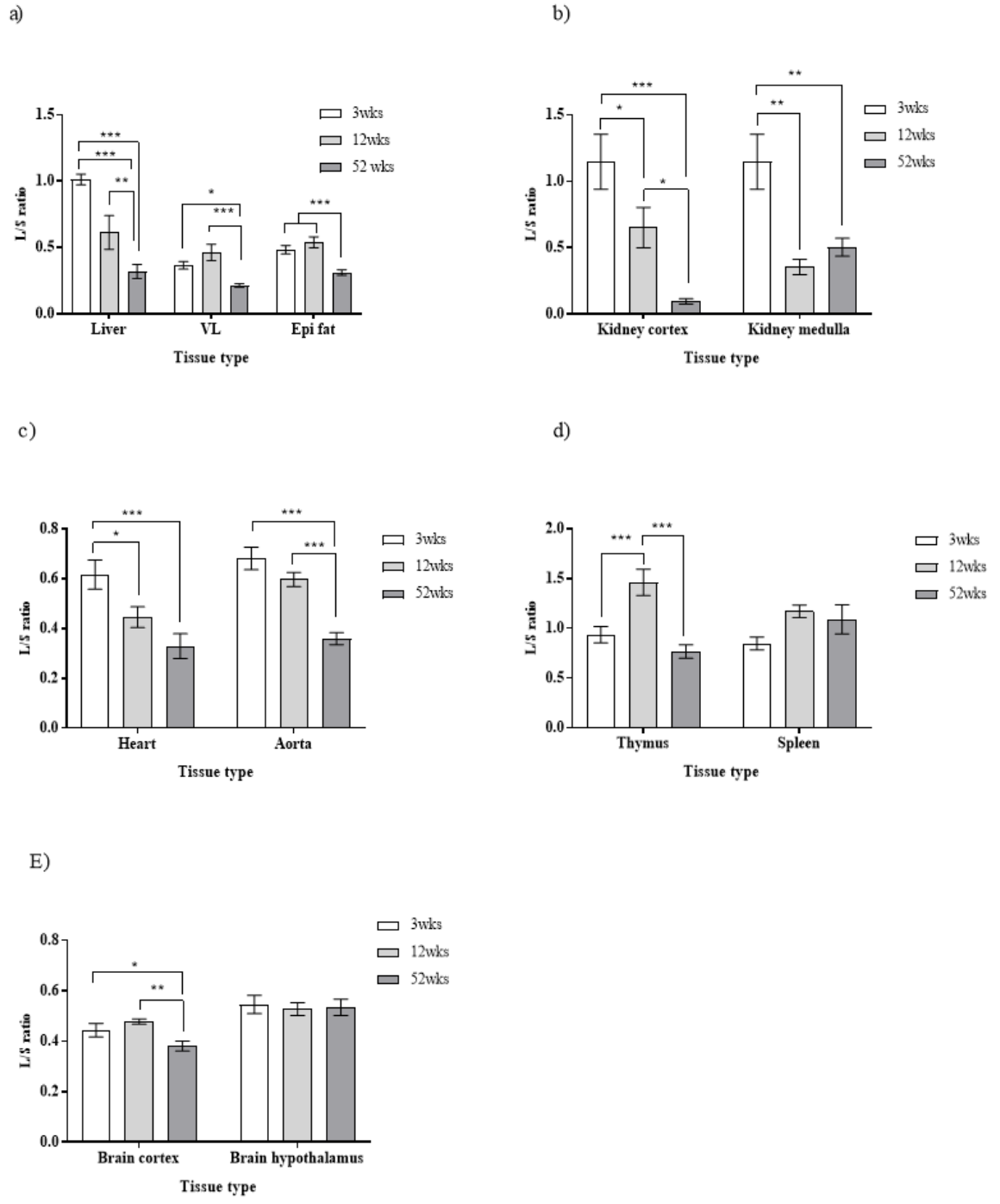
a)
L/S ratio
1.5
1.0
0.5
0.0
***
***
**
*
***
***
3wks
12wks
52 wks
Liver
VL
Epi fat
Tissue type
b)
L/S ratio
1.5
1.0
0.5
0.0
***
*
*
**
**
3wks
12wks
52wks
Kidney cortex
Kidney medulla
Tissue type
c)
L/S ratio
0.8
0.6
0.4
0.2
0.0
***
*
***
***
3wks
12wks
52wks
Heart
Aorta
Tissue type
d)
L/S ratio
2.0
1.5
1.0
0.5
0.0
***
***
3wks
12wks
52wks
Thymus
Spleen
Tissue type
E)
L/S ratio
0.8
0.6
0.4
0.2
0.0
*
**
3wks
12wks
52wks
Brain cortex
Brain hypothalamus
Tissue type

Table 1 Primer sequences

Gene	Forward Sequence	Reverse sequence	Product size (bp)
Tert1	5’ CTGGCAGGTATACGGCTTTC 3’	5’ CTTTAAGAAGCGGCGCTCA 3’	92
Terc	5’ CCGCCGTGAAGAGCTAGT 3’	5’ TCAGGGACCAGTTCCGTTAC 3’	97
Ppia	5’ TGAGAACTTCATCCTGAAGCATACA 3’	5’ CATTTGTGTTTGGTCCAGCATT 3’	89

Table 2 Morphometric analysis of ageing male rats

Tissue	**3 weeks**	**12 weeks**	**52 weeks**
Body weight (g)	49 ± 2 ***	475 ± 15 $$$	956 ± 41 &
Aorta (mg)	11 ± 1 ***	63 ± 11 $$$	85 ± 7 &&&
Heart (mg)	37 ± 2 ***	1436 ± 85 $$$	2055 ± 164 &&&
Kidney (L) (mg)	30 ± 1 ***	1800 ± 63 $$$	2533 ± 163 &&&
Liver (mg)	178 ± 7 ***	17345 ± 758 $$$	28608 ± 2041 &&&
Thymus (mg)	12 ± 1 ***	753 ± 55 $$$	1197 ± 96 &&&
Spleen (mg)	26 ± 4 ***	1129.0 ± 67 $$$	1735 ± 116 &&&
Brain (mg)	144 ± 2 ***	2050 ± 34 $$$	2181 ± 38 &&
VL (mg)	45 ± 11 ***	4115 ± 145 $$$	6691 ± 373 &&&
Epi fat (mg)	-	8396 ± 895	23458 ± 2206 &&&

S1 Table House-keeper data for *Ppia* gene expression

Tissue	**3 weeks**	**12 weeks**	**52 weeks**	**p value**
Liver	36370 ± 2804	34551 ± 3580	36968 ± 1956	0.81
Heart	27888 ± 1150	29805 ± 3595	26814 ± 3033	0.71
Aorta	87980 ± 9303	88429 ± 10767	76261 ± 8227	0.59
Kidney cortex	104751 ± 9170	82542 ± 8386	105054 ± 8587	0.17
Kidney Medulla	107712 ± 12932	96378 ± 8215	124858 ± 12688	0.25
Thymus	53154 ± 5671	48906 ± 9007	46596 ± 6901	0.83
Spleen	16721 ± 3628	15330 ± 1772	19905 ± 3056	0.51